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Placental immune response to apple allergen in allergic mothers

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Abstract

Introduction: The immunological milieu in the placenta may be crucial for priming the developing fetal immune system. Early dysbalances may promote establishment of immune-mediated diseases in later life including allergies. The initial exposure to allergens seems to occur *in utero*, but little is known about allergen induced placental cytokine and chemokine release.

Objectives: The release of several cytokines and chemokines from placenta tissue after exposure to mast cell degranulator compound 48/80 or apple allergen in placentas from allergic and healthy mothers should be analyzed.

Methods: Four placentas from women with apple allergy and three controls were applied in a placenta perfusion model with two separate cotyledons simultaneously perfused with and without apple allergen (Mal d 1). Two control placentas were perfused with compound 48/80. In outflow, histamine was quantified spectrophoto-fluorometrically, IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ by a cytometric multiplex bead array and IL-13 and CXCL10, CXCL11, CCL17 and CCL22 with an in-house multiplex Luminex assay.

Results: Compound 48/80 induced a rapid release of histamine, CXCL10, CXCL11, CCL17 and CCL22, but not of the other factors. Apple allergen induced a time-dependent release of IL-6 and TNF, but not of histamine, in placentas of women with apple allergy as compared to the unstimulated cotyledon. CCL17 levels were slightly increased after allergen stimulation in control placentas.

Conclusion: Allergens can induce placental cytokines and chemokines distinctly in allergic and healthy mothers. These mediators may affect the prenatal development of the immune system and modify the risk for diseases related to immune disorders in childhood such as allergies.

Introduction

The prevalence of allergic diseases has increased during the last decades (Burr *et al.* 1989, Asher *et al.* 2006). Genetic factors are important for allergy development, but a time period of 30-40 years is considered to be to short for human genetic composition to undergo such dramatic changes causing this increasing prevalence. As a consequence, a lot of attention has been drawn to the postnatal exposure to environmental factors associated with a westernized lifestyle. Exposure to environmental factors important for allergy development appears to be important very early in life, perhaps even before birth (Jenmalm and Bjorksten 1998). This concept was first developed in 1989, when D. J. P. Barker highlighted the possible link between events in utero and development of diseases in adult life, called “fetal programming of diseases” (Barker *et al.* 1989). Prenatal farm exposure reduces the risk of asthma symptoms, allergic rhinoconjunctivitis and eczema (Douwes *et al.* 2008) and maternal exposure to stables during pregnancy protects against allergic sensitization, whereas exposures later in life has limited or no effect at all (Ege *et al.* 2006, Lampi *et al.* 2011). The role for the gestational environment on the shaping or immune responses in the offspring and development of allergic diseases needs further investigation, however.

The initial exposure to allergens may occur *in utero*. House dust mite allergen has been detected in the amniotic fluid and in the fetal circulation, indicating a transamniotic and a transplacental transfer (Holloway *et al.* 2000). Dual perfusion experiments have shown a maternal-fetal passage of β-lactoglobulin, ovalbumin and birch pollen (Loibichler *et al.* 2002, Edelbauer *et al.* 2003, Edelbauer *et al.* 2004) but also an accumulation of allergen in the syncytiotrophoblast cell layer (Szepfalusi *et al.* 2006). Detectable allergen-specific T cell responses at birth, shown as a capability

of cord blood mononuclear cells (CBMC:s) to produce cytokines in response to allergens, support the idea of intrauterine allergen exposure and priming of the fetal immune system (Kondo *et al.* 1998, van der Velden *et al.* 2001). On the other hand, the neonatal CD4+ T cell population has shown a typical phenotype of recent thymic emigrants, with receptors lacking the specificity of conventional T cells and may thus be capable to interact with a multitude of antigens, *i.e.* allergens (Thornton *et al.* 2004).

Human term placenta consists of several cell populations including fibroblasts, smooth muscle cells, endothelial cells, cyto- and syncytiotrophoblast cells and immune cells such as macrophages, T cells and mast cells. Many of these cells are able to produce cytokines and chemokines, but macrophages, endothelial cells and trophoblast cells can be accounted for the major production (Steinborn *et al.* 1998, Keelan *et al.* 1999). The chemokines function as attractants for leukocytes to the site of inflammation and the regulation of leukocyte maturation (Pease and Williams 2006). The interleukin (IL)-4 and IL-13 induced chemokines CCL17 and CCL22 (Andrew *et al.* 1998, Nomura *et al.* 2002) bind to the CCR4 receptor expressed on Th2 lymphocytes, mast cells, dendritic cells and natural killer T (NKT) lymphocytes (Pease and Williams 2006). The interferon-γ (IFN-γ) induced chemokines CXCL10 and CXCL11 (Luster and Ravetch 1987, Cole *et al.* 1998) attract CXCR3 receptor expressing Th1 lymphocytes, NKT and mast cells (Pease and Williams 2006). Although allergy is associated with increased allergen induced levels of IL-4, IL-5, IL-13, CCL17 and CCL22 by peripheral mononuclear cells (PBMCs) (Imada *et al.* 1995, Till *et al.* 1997a, Till *et al.* 1997b, Sun *et al.* 2007), little is known about the allergen induced cytokine and chemokine production at the local level in the placenta. Furthermore, allergen induced mast cell degranulation in the placenta has not been

demonstrated. A pronounced placental Th2 shift in allergic mothers has been suggested to explain the greater risk of maternal allergy as compared to paternal allergy for development of allergic diseases in the offspring (Ruiz *et al.* 1992, Liu *et al.* 2003). Furthermore, the higher cord blood (CB) IgE levels in children of allergic mothers than children with paternal or no allergic history (Johnson *et al.* 1996, Liu *et al.* 2003) support a possible exaggerated placental Th2 phenotype among the allergic women. Exposure to a strong Th2 milieu during fetal development could generate long lasting effects in the offspring by modulation of their immune responses, to an IgE favouring, Th2-like phenotype, possibly promoting allergy development later in life.

The aim of the present study was to analyze the cytokines IL-2, IL-4, IL-6, IL-10, IL-13, IFN- γ, Tumor necrosis factor (TNF), the chemokines CXCL10, CXCL11, CCL17, CCL22 and histamine release in placentas after stimulation with apple allergen or the mast cell degranulating compound 48/80 in relation to maternal allergic disease.

Material and methods

Subjects

Four women with an oral allergy syndrome displaying allergic symptoms to apple and 5 women without any allergic symptoms from the Jena area, region of Thuringia, Germany, were included in the study. The following inclusion criteria were applied: delivery after week 37 of pregnancy, a healthy appropriately grown newborn, absence of maternal chronic metabolic diseases, pharmacological therapy and pregnancy complications. In their anamneses, none of the allergic patients has reported systemic reactions, but only the classical local reactions as described for the oral allergy syndrome (Ortolani *et al.* 1988). The similar severity of described symptoms did not allow subdivision of the patients group. All study participants gave their written informed consent. The regional ethics committee of the Medical Faculty of Friedrich Schiller University Jena approved the study (No. 1038-02/03).

In advance to delivery, circulating allergen specific IgE antibodies to the major allergens of apple (Mal d 1) and birch (Bet v1; because of their cross-reactivity (Klinglmayr *et al.* 2009)) were measured in serum of the allergic women by using specific IgE tests (ImmunoCAP; Phadia, Freiburg, Germany) and a Phadia®250 system. If this was not practicable, a rapid immunographic allergy screening test (Auro Dex Visual-ENS test, including birch, other tree and grass pollen, and frequent animal allergens; Dexall, USA) was conducted in the delivery room. After delivery, results were confirmed by an ImmunoCAP test. Sensitisation to additional allergens did not lead to exclusion. Both of the rapid diagnosis allergy tests were also used to exclude allergic sensitisation in the anamnestically non-allergic women. The sensitivity and specificity of the Auro-Dex Visual-Ens has been assessed previously (Pietsch 2006).

Three of the four allergic women had in our laboratory a positive IgE test to apple or birch (CAP class II-V) and the fourth patient showed a medical certificate for confirmation of sensitisation to apple (CAP class II). CAP classes could not be used for defining subgroups due to the limited availability of placentas from allergic individuals. None of the non-allergic women were sensitised to the analysed allergens.

Isolation of apple allergen (Mal d 1)

Mal d 1 was extracted from fresh apples in the same lab and by using the same protocol as previously published (Rudeschko *et al.* 1995a, Rudeschko *et al.* 1995b). The concentration has been determined as described previously (Vieths *et al.* 1994, Rudeschko *et al.* 1995b). Briefly, apples (Golden Delicious from a local store) were homogenised at 4°C in an extraction buffer containing phosphate-buffered saline (PBS; PAA, Pasching, Austria), Polyvinylpyrrolidon (Sigma-Aldrich, Steinheim, Germany), Ethylenediaminetetraacetic acid (EDTA; Roth, Karlsruhe, Germany), Diethyl-dithiocarbamate (Sigma-Aldrich), Benzamidinhydrochlorid (Sigma-Aldrich) and Phenylmethan-Sulfonyl-chlorid (Sigma-Aldrich), at pH 7.4 using a pH meter (FiveEasy; Mettler-Toledo, Gießen, Germany). The homogenised apples were filtered and dialysed two times against an EDTA - Diethyldithiocarbamate solution and 3 times against a Tris(hydroxymethyl)aminomethane (Tris; Sigma-Aldrich) buffer, pH 8.0. The extract was applied on a Q-Sepharose Fast Flow column (Sigma-Aldrich) and eluted by addition of Tris, pH 8.0. The total concentration of protein in the eluate was determined by a Bradford assay and the concentration of Mal d 1 with ELISA (Heinzelmann 2005). Mal d 1 was lyophilised and stored at -20°C. Two kg apples generated 13 mg Mal d 1. We have produced and used a total two lots of the

above described allergen preparation and both have been used for perfusion of placentas of allergic and non-allergic mothers. The activity and stability of the so produced allergen has been reported in detail previously (Rudeschko *et al.* 1995a, Rudeschko *et al.* 1995b).

One sided placenta perfusion

Placentas were obtained after spontaneous delivery (Allergic women n=3, Non-allergic women n=1) or cesarean section (Allergic women n=1, Non-allergic women n=2). Because of the limited accessibility to placentas from allergic women both groups have been merged. A single sided placenta perfusion system was developed which allows simultaneous separate and independent perfusion of two cotyledons of the same placenta. In all experiments one cotyledon has been perfused with apple allergen or compound 48/80 and the other with control medium. To evaluate the placenta vitality and functionality in this system, we compared metabolic parameters in conditioned perfusion medium with those in dually (fetal and maternal side) perfused placentas as previously done in our laboratories (adapted after (Schneider and Huch 1985)). No significant differences in glucose consumption, lactate production, secretion of β-hCG or consumption of oxygen was detected.

Two cotyledons from each placenta were cut out surrounded by sufficient tissue for fixation in the chambers. On the upside, the maternal tissue was penetrated by four blunt metal cannulae through the decidual plate into the intervillous space. The fetal tissue remained untouched. The perfusion medium consisted of NCTC-135 tissue culture medium (Cambrex, Verviers, Belgium) diluted 2:1with Earl´s-Buffer (Biochrom, Berlin, Germany), and supplemented with bovine serum albumin (40 g/l; MP Biomedicals, Illkirch, France), D-glucose (1,33 g/l; Merck, Darmstadt, Germany),

amoxillin (250 mg/l; Sigma-Aldrich), heparin (500 µl/l, equivalent to 2500 IU/l, Ratiopharm, Ulm, Germany) and dextran FP40 (10 g/l, Serva, Heidelberg, Germany), adjusted to an pH of 7,4 by NaOH (Roth, Karlsruhe, Germany). For perfusion, the medium was warmed up to 37°C and oxygenated by using a Silox-S oxygenator (Mera Senko Medical Instrument, Tokyo, Japan). The flow rate was 2.2 ml/min during the entire perfusion period. The experimental cotyledon was perfused with pure perfusion medium for 1 h followed by medium containing 4 µg/ml Mal d 1 (similar concentrations are able to induce strong basophil activation (Erdmann *et al.* 2005)) or 0.1 mg/ml compound 48/80 (Sigma-Aldrich) for further 4 to 5 h. Alternatively, after 1 h of mock perfusion, compound 48/80 has been applied as a bolus of 30 mg/5 ml directly via the influx tubes into the placenta. The control cotyledon was perfused with pure perfusion medium up to 6 hours. To monitor the metabolic state, pH, pO_2 und pCO_2 were analysed every 30 minutes in arterial and venous flow. Venous outflow from both cotyledons was collected in 10 minutes steps for further analysis. To remove remaining tissue fragments, samples were centrifuged for 10 min, at 3500 g and 4° C. Supernatants were stored in aliquots at -20° C until analysis.

Spectrophoto-fluorometrically quantification of histamine

The histamine concentration in the perfusion outflow from the experimental and the control cotyledon of 2 placentas stimulated with compound 48/80 and 2 placentas stimulated with Mal d 1 was measured spectrophoto-fluorometrically as described in detail elsewhere (Shore *et al.* 1959, Ronnberg and Hakanson 1984). The extraction protocol and the excitation wave lengths were adapted to the analysis of perfusion medium. Histamine was extracted from 0.5 ml perfusion medium by using a mixture of 0.5 ml 0.9% NaCl (Roth), 2.5 ml n-butanol (Roth) and 0.2 ml 3 M NaOH. After 3

min incubation on a shaker, the mixture was centrifuged at 900 g for 20 min. Two ml of the butanol-phase was removed and mixed with 1.2 ml 0.12 M HCl and 3.8 ml n-heptan (Roth). After 1 min incubation on a shaker, followed by a centrifugation at 600 g for 5 min, the n-heptan-phase was removed, cooled on ice, and mixed with 0.4 ml 0.75 M NaOH (Roth) and 0.12 ml methanolic o-phthalaldehyde (OPT; Sigma-Aldrich). After 4 min incubation, the reaction was terminated by addition of 0.2 ml 2 M H_3PO_4 on ice. The fluorescence was measured at λEx=355nm; λEm=440nm using the Fluorescenence HPLC Monitor RF_551 (Shimadzu, Duisburg, Germany) with 40% MeOH/aqua dest. as eluent. The fluorescence data were acquired and calculated using Chromeleon-software (Dionex, Germering, Germany). The standard curve was done in duplicates and ranged from 0.5 to 50 ng/ml (dilution steps: 0; 0.5; 1; 3; 5; 10; 20; 50 ng/ml; $R^2 > 0.998$) and revealed a lower detection limit of 5 ng/ml. The coefficient of variance (CV) was below 1.5%.

Quantification of IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ using a cytometric multiplex bead array

The cytokines IL-2, IL-4, IL-6, IL-10, TNF and IFN- γ were measured in the conditioned perfusion medium by using a cytometric multiplex bead array following the manufacturer's kit instructions (Human Th1/Th2 Cytokine Kit II, BD Bioscience, Heidelberg, Germany).

Briefly, the samples (perfusion medium or standard) were mixed (1:1:1) with antibody-coated fluorescent beads (λEm=670nm) and the R-phycoerythrin conjugated detection antibodies (λEm=575nm), and incubated shaking for 3 hours at room temperature in the dark. For standard, recombinant proteins (IL-2, IL-4, IL-6, IL-10, TNF and IFN- γ; from assay kit) were dissolved in buffer medium and serially

diluted (1:1) from 5000 to 20 pg/ml for each cytokine. The beads were washed, spinned down (200 g, 5 min), and after removement of supernatants, measured on a flow cytometer (FACS Calibur; BD Bioscience) by using the implemented FCAP Array v1.0.1. kit software with 5-parametric-curve fitting. The sensitivity limits were 2.6 pg/ml for IL-2, 2.6 pg/ml for IL-4, 3 pg/ml for IL-6, 2.8 pg/ml for IL-10, 2.8 pg/ml for TNF and 7.1 pg/ml for IFN-γ. The CV was below 10%.

Determination of CXCL10, CXCL11, CCL17, CCL22 and IL-13 by an in-house multiplex Luminex assay

The levels of CXCL10, CXCL11, CCL17, CCL22 and IL-13 in the perfusion medium were measured using an in-house multiplex Luminex assay, as described in detail elsewhere (Abrahamsson *et al.* 2011). Briefly, the monoclonal anti-human CXCL10 (clone 4D5, BD Biosciences, Stockholm, Sweden), CXCL11 (clone 87328, R&D Systems, Abingdon, UK), CCL17 (clone 54026, R&D Systems), CCL22 (clone 57226, R&D Systems) and IL-13 (Ref: M191302, Sanquin, Amsterdam, The Netherlands) antibodies were covalently coupled to carboxylated microspheres at a concentration of 5 µg antibody/10^6 microspheres, using the protocol recommended by the manufacturer (Luminex Corporation, Austin, TX, USA). 2000 coupled microspheres were added to each well of a 1.2 µm pore-size filter plate (Millipore multiscreen, Millipore Corporation, Bedford, USA) and incubated over night with either recombinant human CXCL10, CXCL11, CCL17, CCL22 and IL-13 (R&D Systems), or samples diluted 1:2. The microspheres were washed, incubated for 1 h with biotinylated anti-human CXCL10 (1000 ng/ml, clone 6D4, BD Biosciences), CXCL11 (500 ng/ml, BAF320, R&D Systems), CCL17 (500 ng/ml, BAF364, R&D Systems), CCL22 (200 ng/ml, BAF336, R&D Systems) and IL-13 (200 ng/ml, Ref: M191304,

Sanquin) antibody, followed by incubation with 1 µg/ml Streptavidin R-phycoerythrin conjugate (Molecular Probes, Eugene, USA) for 30 minutes. A Luminex^{100} instrument (Biosource, Nivelles, Belgium) was used for analysis of the samples and the data acquisition was performed using the StarStation 2.3 software (Applied cytometry systems, Sheffield, UK) with 5-parametric-curve fitting. The sensitivity limits were 6 pg/ml for CXCL10 and CXCL11, 1 pg/ml for CCL17 and CCL22 and 8 pg/ml for IL-13. The samples were analyzed in duplicates and the CV was below 15%. Undetectable levels were given the value of the half cut-off.

Statistical analysis

Due to the explorative nature of the study and the small sample size the data were primarily analyzed by descriptive methods. To generate hypotheses about the response to allergen exposure over time mixed linear models were applied with time, allergen and their interaction as fixed and subject as random factors. The level of significance was 0.05. The analyses were performed with SAS 9.3 software.

Results

Generally, in most cases the analytes in the perfusate decrease during the first hour of placenta perfusion. The values at timepoint 0 represent the concentrations at the very beginning of perfusion and may be similar to serum concentrations. The first hour of perfusion is performed with pure medium without a stimulus, which usually reduces the concentrations.

Compound 48/80 induced histamine and chemokine release

To determine if mast cells in the placenta are able to degranulate, two placentas from non-allergic mothers were perfused with compound 48/80. When this was added as a bolus to the placenta 60 minutes after the beginning of perfusion, a strong histamine release was immediately detectable (Fig 1). After this rapid response to compound 48/80, the histamine levels decreased to basis level, followed by a second increase of histamine release after 3 hours, albeit no further compound 48/80 was added. The chemokines CXCL10, CXCL11, CCL17 and CCL22 (Fig 2A-D) were also released rapidly after mast cell activation with compound 48/80, but not IL-6 (Fig 2E) and TNF (Fig 2F).

When 0.1 mg/ml compound 48/80 was permanently added to the perfusion medium, histamine levels were very low and only sporadically detectable during the analysed time period. In analogy to the bolus application, at the initiation of perfusion with compound 48/80, a rapid release of the chemokines CXCL10, CXCL11, CCL17 and CCL22, but not of the cytokines IL-2, IL-4, IL-13, IFN-γ, IL-6 and TNF was observed (data not shown).

Allergen induced cytokine and chemokine release

The activity and stability of the apple allergen preparation has been demonstrated previously by performing series of analyses including SDS-PAGE, two-dimensional electrophoresis, immunoblotting, RAST inhibition, and prick test (Rudeschko *et al.* 1995a, Rudeschko *et al.* 1995b). Perfusion of placentas with apple allergen induced a time-dependent increase of IL-6 (Fig 3A) and TNF (Fig 3B) as compared to the unstimulated cotyledon, in placentas of women with apple allergy. The stronger increase after stimulation could be confirmed by a significant interaction between time and allergen for TNF ($F_{(1,125)}$=16.6, p<0.001) and IL-6 ($F_{(1,116)}$=25.1, p<0.001) in the mixed model analyses. IL-6 and TNF were also released spontaneously from placentas of non-allergic women, but without further increase after instillation of apple allergen. The CCL17 levels were slightly elevated after allergen stimulation in placentas of women without apple allergy (Fig 3C), but the effect was not statistically significant (detailed results in supplementary table 1). The secreted levels of CXCL10, CXCL11 and CCL22 from the experimental and control cotyledon were similar in both groups (data not shown), and the levels of IL-2, IL-4, IL-13 and IFN-γ were undetectable or only sporadically detectable in the samples. Histamine release was not induced by stimulation with apple allergen (all results summarized in table 1).

Discussion

This study has been performed to test if in patients with an oral allergy syndrome, allergen challenge of the placenta induces release of histamine, cytokines or chemokines. As a positive control for the potential of placental mast cell reactivity, placentas have been perfused with compound 48/80. This degranulating stimulus induced histamine secretion, but no detectable effects on the analysed cytokines. On the other hand, apple allergen challenge induced secretion of IL-6 and TNF in placentas from allergic mothers. Therefore, in our system the source of both cytokines seems to be distinct from mast cells, although previous studies have demonstrated that mast cells can release TNF and IL-6 selectively upon stimulation with compound 48/80, PMA or several other stimuli even without simultaneous histamine release (Kruger-Krasagakes *et al.* 1999, Gibbs *et al.* 2001, Kandere-Grzybowska *et al.* 2003, Theoharides and Kalogeromitros 2006, Kim *et al.* 2007, Kulka *et al.* 2008), thus making it inappropriate to completely exclude the ability of mast cells in the placenta to produce these cytokines.

It may be argued that the way of delivery, with or without labor, influences cytokine levels as reported for IL-6, but not TNF in cord blood (Duncombe *et al.* 2010). The intraindividual control perfusion, one cotyledon with and one without allergen, should overcome these discrepancies in basic levels. Levels of both cytokines increase during the course of placenta perfusion and can be seen as stress markers, which have been reported in previous studies (Pierce *et al.* 2002, Di Santo *et al.* 2007). We observed this increase in both groups of allergic and healthy individuals, but the further increase of these two proinflammatory cytokines in response to apple allergen in placentas of allergic mothers indicates an enhanced general inflammatory activity in this group. Inhalant allergens have been shown to induce IL-6 and TNF production

in airway epithelial cells (Vroling *et al.* 2007), alveolar macrophages (Chen *et al.* 2003), monocytes and monocyte-derived macrophages (Andersson Lundell *et al.* 2005), indicating that allergens are able to evoke proinflammatory immune responses as an early response to the allergen. The allergen induced IL-6 and TNF levels from monocytes were independent of LPS contamination, evaluated by adding the LPS-neutralizing agent polymyxin B (Andersson Lundell *et al.* 2005). Placenta perfusion systems are not sterile and although all parts of the system (e.g. tubing) are intensively cleaned and disinfected after each use, potential endotoxin contamination cannot be excluded. Nevertheless, the different responses on allergens (from the same batches) in allergic and non-allergic women, when comparing the allergen perfused and control cotyledons, suggest that the induction of IL-6 and TNF are in response to the allergen rather than to endotoxins.

Even though IL-6 and TNF are not generally considered as strong inducers of Th2-associated immune responses, house dust mite stimulated alveolar macrophages from mice promote T cell proliferation and Th2-cell development by up-regulation of costimulatory B7 molecules and secretion of IL-6 and TNF, indicating a possible role for these proinflammatory cytokines in the allergic inflammation (Chen *et al.* 2003). Maternal allergy did not correlate with elevated Th2-like chemokine responses to apple allergen in our model, but a diminutive allergen induced increase of CCL17 levels was observed in placentas of non-allergic women. These findings do not necessary exclude the possibility of enhanced Th2-like responses to allergens in the placentas of allergic women, as the signature cytokines of a Th2-like immunity, IL-4 and IL-13, were undetectable in the majority of the samples. Thus, low levels of these Th2-like cytokines might be induced, but, due to methodological limitations, impossible to detect. CCL17 and CCL22 are readily detectable in the human

circulation, probably explaining the high chemokine levels in the beginning (time point 0) of the perfusion experiment (Fig 3A-D). Only approximately 50% of the samples had detectable levels of CCL17 during the course of perfusion, which indicates that its physiological source is outside the placenta.

Mast cells contribute to the allergic inflammation by the release of granule-mediated substances such as histamine by an FcεRI-dependent pathway, whereas mast cell activation by FcεRI-independent pathways such as Toll like receptor signalling, stimulation with components from the complement system, cytokines and chemokines, may be implicated in various innate and adaptive immune responses (Metcalfe *et al.* 1997, Menzies *et al.* 2011). The present study demonstrates the kinetics of histamine expression from placental mast cells on specific activation by compound 48/80, which indicates their potential for classical allergen-induced inflammatory reactions. A previous study has indirectly reported histamine release from placental mast cells by demonstrating a decrease of histamine in placenta tissue after 90 minutes perfusion with atrial natriuretic peptide (Szukiewicz *et al.* 2001). Degranulation of placental mast cells has also been described after stress induced substance P increase in murine placentas (Markert *et al.* 1997). Isolated uterine mast cells secrete histamine through the FcεRI-dependent pathway in response to anti-IgE stimulation (Massey *et al.* 1991). IgE is present in the human placenta: in the maternal as well as in the fetal tissue in women with, but also without, allergies (Rindsjo *et al.* 2010).The placenta IgE levels correlate with those in blood (Joerink *et al.* 2009). Therefore, we did not reproduce these experiments, as all patients in our analysis had IgE to Mal d 1 and Bet v 1 in their serum. Histamine is an important mediator in the course of pregnancy, in particular during labour by inducing contractions of the myometrium, both directly and indirectly by inducing

prostaglandin production, indicating that mast cell degranulation in the reproductive tract needs to be strictly regulated (reviewed in (Menzies *et al.* 2011)). In the here applied placenta perfusion system, histamine release was not induced by tissue perfusion with apple allergen. It may be argued that too little bioactive apple allergen concentrations have reached the mast cells. This may be due to dilution of allergens or to filter effects of tissue barriers, but also to influences on the stability of apple allergen such as by medium or tissue components (Rudeschko *et al.* 1995b). As allergens may appear via the circulation in the placenta, it is tempting to speculate that presence of a high proportion of mast cells with allergen specific IgE antibodies attached to FcεRI in the placenta, may confer a risk for preterm labour. Maternal allergy has been associated with longer gestational age, higher birth weight (Somoskovi *et al.* 2007) and less pre-term births (Savilahti *et al.* 2004), indicating favourable effects on the maintenance of pregnancy rather than detrimental effects, but the role of histamine in the underlying mechanisms is not known. The presence of maternal IgE in the placenta has been summarized recently. It was mainly detected around fetal Hofbauer cells in the villi, but little is known about its binding on mast cells (Rindsjo *et al.* 2010).

The IgE- and FcεRI-independent mechanism for mast cell degranulation by compound 48/80 is not determined, but an effect on the plasma membrane has been suggested, for example through interactions with different types of receptors, membrane transporters and translocation across the membrane (Ferry *et al.* 2002). Subsequent signalling through the G protein coupled receptors Mas related gene X1 (MrgX1) and MrgX2 have been suggested (Tatemoto *et al.* 2006, Kashem *et al.* 2011). We could demonstrate that mast cells in the placenta are able to synthesise and secrete chemokines upon activation, as compound 48/80 induced release of

CXCL10, CXCL11, CCL17 and CCL22 in the present study. Cytokines and chemokines are in general considered to be de novo synthesised upon mast cell activation (Kalesnikoff and Galli 2008, Menzies *et al.* 2011). On the other hand, mast cells and basophils can release preformed, as well as newly synthesised, TNF (Gordon and Galli 1991, Gibbs *et al.* 2001, Kulka *et al.* 2008), IL-4 (Gibbs *et al.* 1996), IL-6 (Kruger-Krasagakes *et al.* 1999, McCall-Culbreath *et al.* 2011) and CXCL8 (Gibbs *et al.* 2001) following activation. The chemokines CXCL10, CCL17, CXCL11 and CCL22 were released rapidly, only 20 minutes after addition of compound 48/80. The latter two remained steadily secreted during the entire perfusion experiment (Fig 3B and 3D), whereas after approximately 3 h, the concentrations of CXCL10 (Fig 3A) and CCL17 (Fig 3C) have decreased to levels similar to the control cotyledon. Thus, our data possibly indicate a rapid release of preformed chemokines, followed by a continuous release of newly synthesized CXCL11 and CCL22.

In conclusion, allergen induced mast cell degranulation, cytokine and chemokine responses may occur in the placenta. As previously summarized, these reactions can shape or prime infant immune development. The increased allergen induced IL-6 and TNF levels in placentas of allergic women as compared to non-allergic women, indicate enhanced proinflammatory immune responses to apple allergen in the allergic group, potentially influencing the shaping of immune responses in the offspring. This observation may contribute to explain the elevated risk of newborns from allergic mothers under allergen exposure for developing allergies in later life.

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Table 1

Table 1. A summary of the induced analytes after Compound 48/80 or Mal d 1 stimulation.

	Compound 48/80	**Mal d 1**	**Mal d 1**
Analyte	Non-allergic, n=2	Allergic, n=4	Non-allergic, n=3
Histamine	**+**	**-** (n=2)	Not analysed
IL-6	**-**	**+**	**-**
TNF	**-**	**+**	**-**
CXCL10	**+**	**-**	**-**
CXCL11	**+**	**-**	**-**
CCL17	**+**	**-**	**+**
CCL22	**+**	**-**	**-**

+; The production of the analyte was induced by stimulation with Compound 48/80 or Mal d 1 as compared to the unstimulated cotyledon.
-; The production of the analyte was not induced by stimulation with Compound 48/80 or Mal d 1 as compared to the unstimulated cotyledon.

Figure legends

Figure 1. Two cotyledons of a human placenta from a non-allergic woman have been simultaneously and independently perfused for 320 min. After 1 h, 30 mg compound 48/80 was injected into the medium of one cotyledon (grey bars), while the other was exclusively medium pefused for control (white bars). The histamine concentration has been analysed in the placenta outflow medium in 20 min steps and has been calculated as ng histamine/ml perfusion medium/kg placenta tissue.

Figure 2. Two cotyledons of a human placenta from a non-allergic woman have been simultaneously and independently perfused for 320 min. After 1 h, 30 mg compound 48/80 was injected into the medium of one cotyledon (permanent line), while the other was exclusively medium pefused for control (broken line). Chemokine (**A**: CXCL10; **B**: CXCL11; **C**: CCL17; **D**: CCL22) and cytokine (**E**: IL-6; **F**:TNF) concentrations have been quantified by cytometric bead arrays in the placenta outflow medium at several time points (dots) and calculated as pg chemokine or cytokine/ml perfusion medium/kg placenta tissue.

Figure 3. Two cotyledons of human placentas from women with and without apple allergy have been simultaneously and independently perfused for up to 360 min. Beginning after 1 h, one cotyledon was perfused with medium containing 4 µg/ml Mal d 1 apple allergen (permanent line), while the other was exclusively medium pefused for control (broken line). IL-6, TNF and CCL17 concentrations have been quantified by cytometric bead arrays in the placenta outflow medium at several time points (dots) and calculated as pg/ml perfusion medium/kg placenta tissue. The spontaneous secretion of these factors during the course of perfusion has been

reported previously and can be seen as the individual baseline for each placenta (Di Santo *et al.* 2007). For visual clarity only medians are presented (for detailed data see supplementary table 1). By applying mixed model analyses, for TNF ($F_{(1,125)}$=16.6, p<0.001) and IL-6 ($F_{(1,116)}$=25.1, p<0.001) the stronger increase after stimulation could be confirmed by a significant interaction between time and allergen.

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Table

Time / Analyte	0 Min →	30	60	80	100	120	140	160	180	200	220	240	260	280	300	320	340	360
TNF	192	50	81	53	94	141	215	326	467	581	745	918	771	1068	1278	1728	3496	1921
A, +Al	38	0	30	45	65	108	186	132	203	397	278	315	206	349	540	311	550	1921
	269	291	248	392	306	537	756	1995	1722	766	5537	4731	5999	5688	5873	8213	6955	1921
TNF	80	124	119	117	114	126	134	102	103	169	231	436	250	317	409	450	479	342
A, -Al	19	55	58	40	80	47	56	86	68	118	155	156	120	182	157	149	132	185
	391	258	345	387	336	258	391	267	623	543	345	1123	3384	2111	3001	3549	3763	499
TNF	45	105	89	64	89	105	234	365	386	448	642	1068	1354	1311	1789	1825	2975	2989
NA, +Al	45	43	40	52	63	74	126	242	342	383	509	1029	1006	1311	738	1789	1444	2812
	120	129	147	243	454	528	978	1205	1690	2350	2187	3031	4066	1311	4747	1860	4506	3165
TNF	69	65	143	131	140	152	405	318	311	417	682	1076	1268	1568	1691	2395	3574	2898
NA, -Al	38	0	57	39	62	133	110	180	245	311	480	988	1112	1100	930	1509	1661	1469
	147	129	228	224	342	443	838	1184	1480	1452	2294	2430	2648	1724	3887	3282	5486	4327
IL-6	1605	356	402	365	243	371	482	298	560	418	539	1122	799	1602	2831	4474	7156	13105
A, +Al	1595	197	213	181	235	323	303	293	334	418	482	494	644	713	1403	852	1310	3554
	2182	625	617	665	436	662	484	487	968	418	951	4785	2713	9655	13219	22504	21469	21571
IL-6	1674	497	518	291	320	310	198	270	333	207	354	834	383	710	1026	1260	1700	2992
A, -Al	1207	458	168	247	243	193	183	149	137	130	190	284	280	372	378	439	359	439
	6236	536	884	848	364	880	213	288	1002	410	431	1691	706	1781	2982	4475	4475	5736
IL-6	877	353	230	212	245	461	407	624	877	809	1519	2824	4275	4019	5283	7053	7850	11954
NA, +Al	617	139	140	174	164	144	274	444	515	613	1214	2018	2170	3088	4131	5335	6318	9030
	1865	628	619	713	777	637	1146	1190	1662	2350	2678	4840	6390	4950	9837	8771	9383	14878
IL-6	1455	580	435	400	376	609	440	560	553	807	1403	2731	4035	3917	4589	6008	6582	6777
NA, -Al	544	201	160	165	188	188	390	532	538	781	1073	1364	1818	1743	2018	3760	4157	3487
	2522	958	711	635	789	782	1186	1623	2121	2081	3680	5777	5391	4017	10884	8255	9008	10068
CCL17	407	-	-	46	-	40	-	37	-	32	-	29	-	14	-	31	31	28
A, +Al	223	-	-	10	-	10	-	10	-	10	-	10	-	9	-	10	10	10
	792	-	-	383	-	230	-	285	-	49	-	45	-	45	-	45	45	47
CCL17	273	-	-	37	-	54	-	49	-	33	-	13	-	13	-	13	13	9
A, -Al	16	-	-	10	-	10	-	10	-	10	-	9	-	9	-	9	9	9
	1493	-	-	288	-	249	-	230	-	230	-	57	-	230	-	57	57	10
CCL17	325	-	-	136	-	119	-	56	-	88	-	99	-	17	-	112	100	135
NA, +Al	232	-	-	82	-	17	-	17	-	17	-	17	-	14	-	88	70	128
	951	-	-	203	-	209	-	200	-	150	-	118	-	109	-	136	130	143
CCL17	450	-	-	113	-	28	-	21	-	21	-	21	-	19	-	17	17	17
NA, -Al	316	-	-	88	-	21	-	16	-	16	-	16	-	16	-	16	16	16
	782	-	-	232	-	215	-	128	-	108	-	88	-	21	-	19	19	19

Supplementary table 1.

TNF, IL-6 and CCL17 levels secreted from placentas of allergic and non-allergic women with and without allergen stimulation.

The table shows the median (first row), minimum (second row) and maximum (third row) levels of TNF, IL-6 and CCL17 in the placenta outflow medium divided to the cotyledon size ((pg/ml)/kg) from placentas of allergic and non-allergic women with and without allergen stimulation.

A=Allergic women, NA=Non-allergic women, +Al=with allergen, -Al=without allergen, min=minutes

Figure

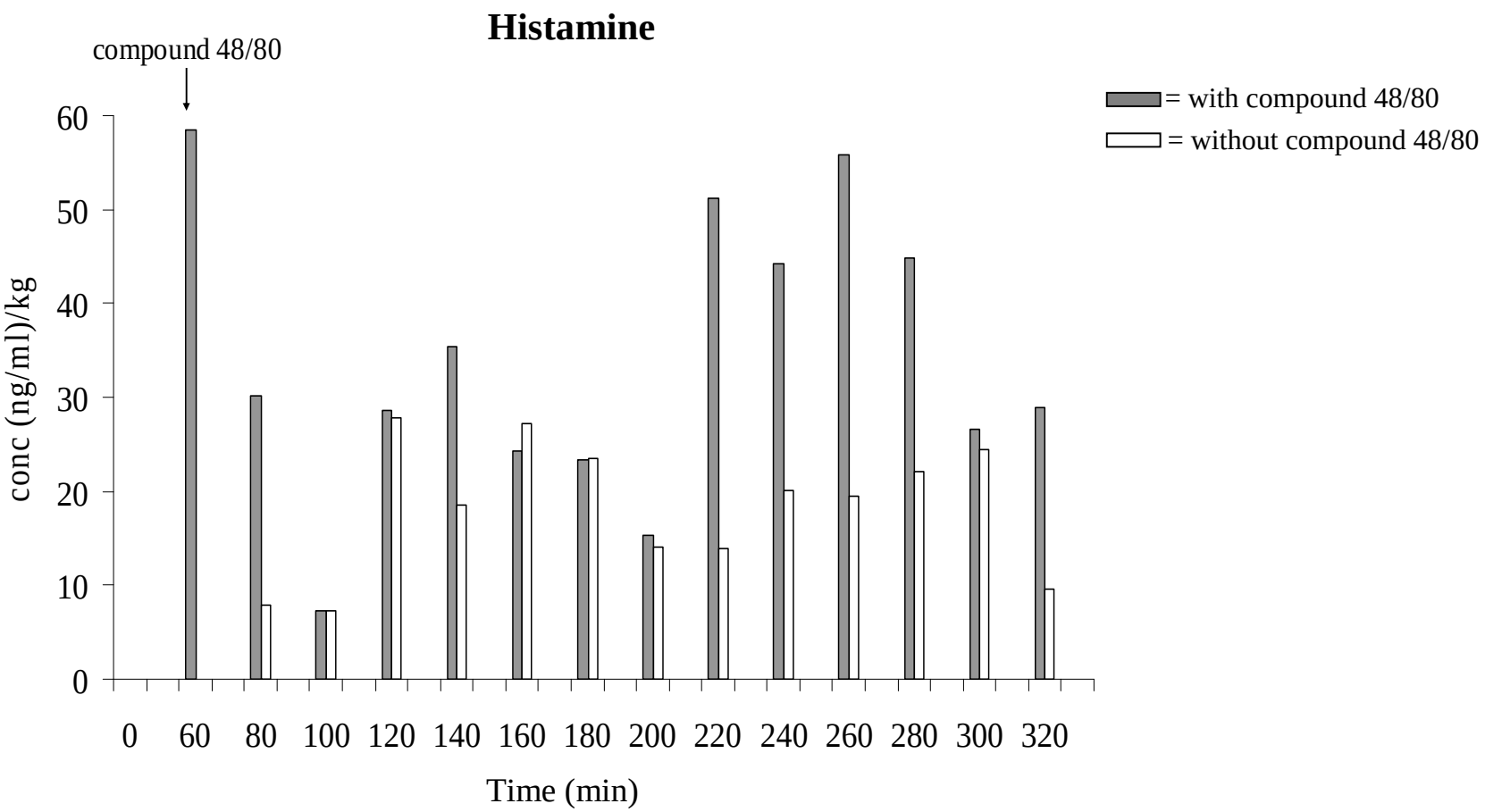
Histamine
compound 48/80
= with compound 48/80
= without compound 48/80
conc (ng/ml)/kg
0
10
20
30
40
50
60
0
60
80
100
120
140
160
180
200
220
240
260
280
300
320
Time (min)

Fig 1

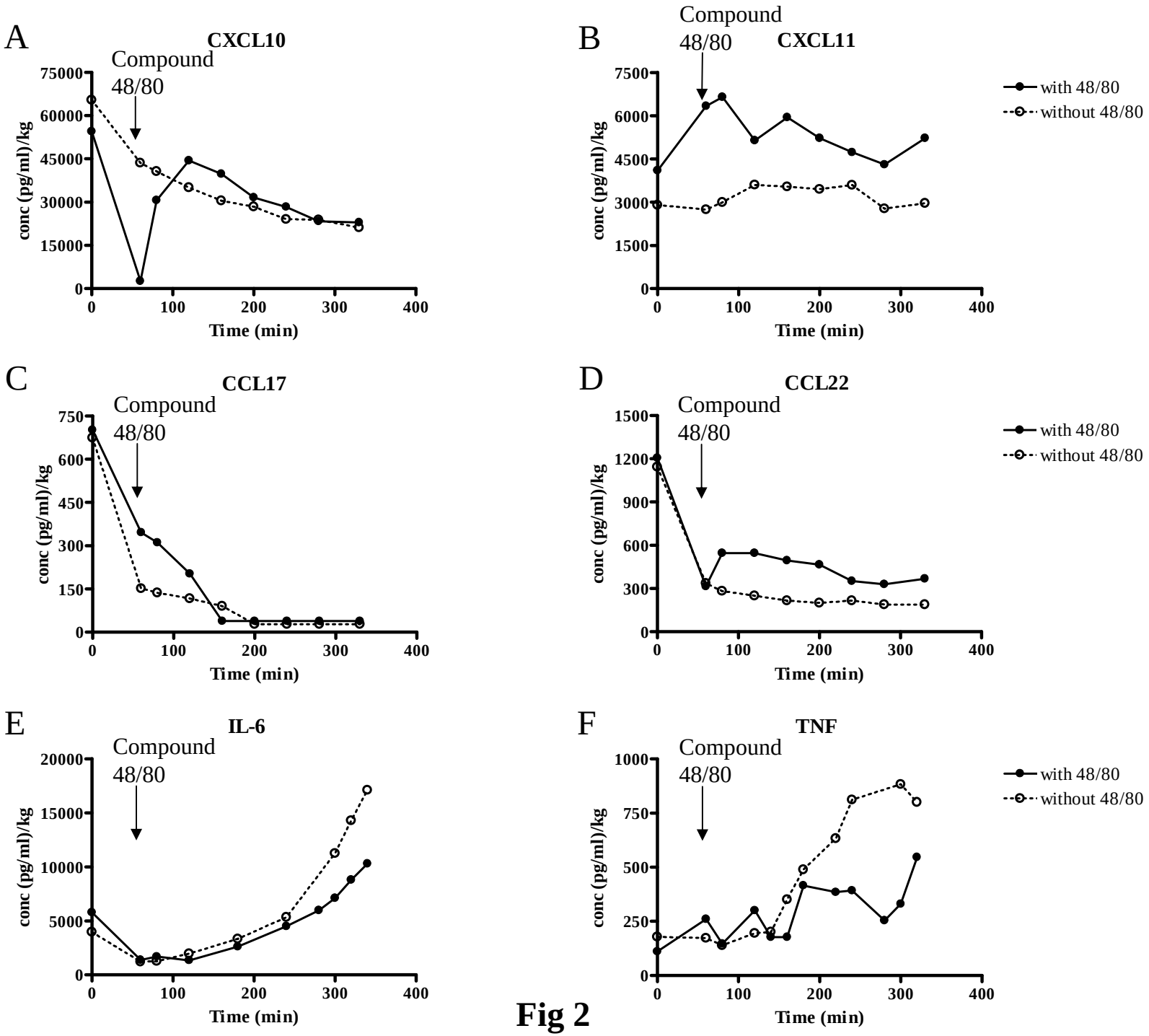
A
CXCL10
Compound
48/80
conc (pg/ml)/kg
Time (min)
B
CXCL11
Compound
48/80
with 48/80
without 48/80
C
CCL17
D
CCL22
E
IL-6
F
TNF

Fig 2

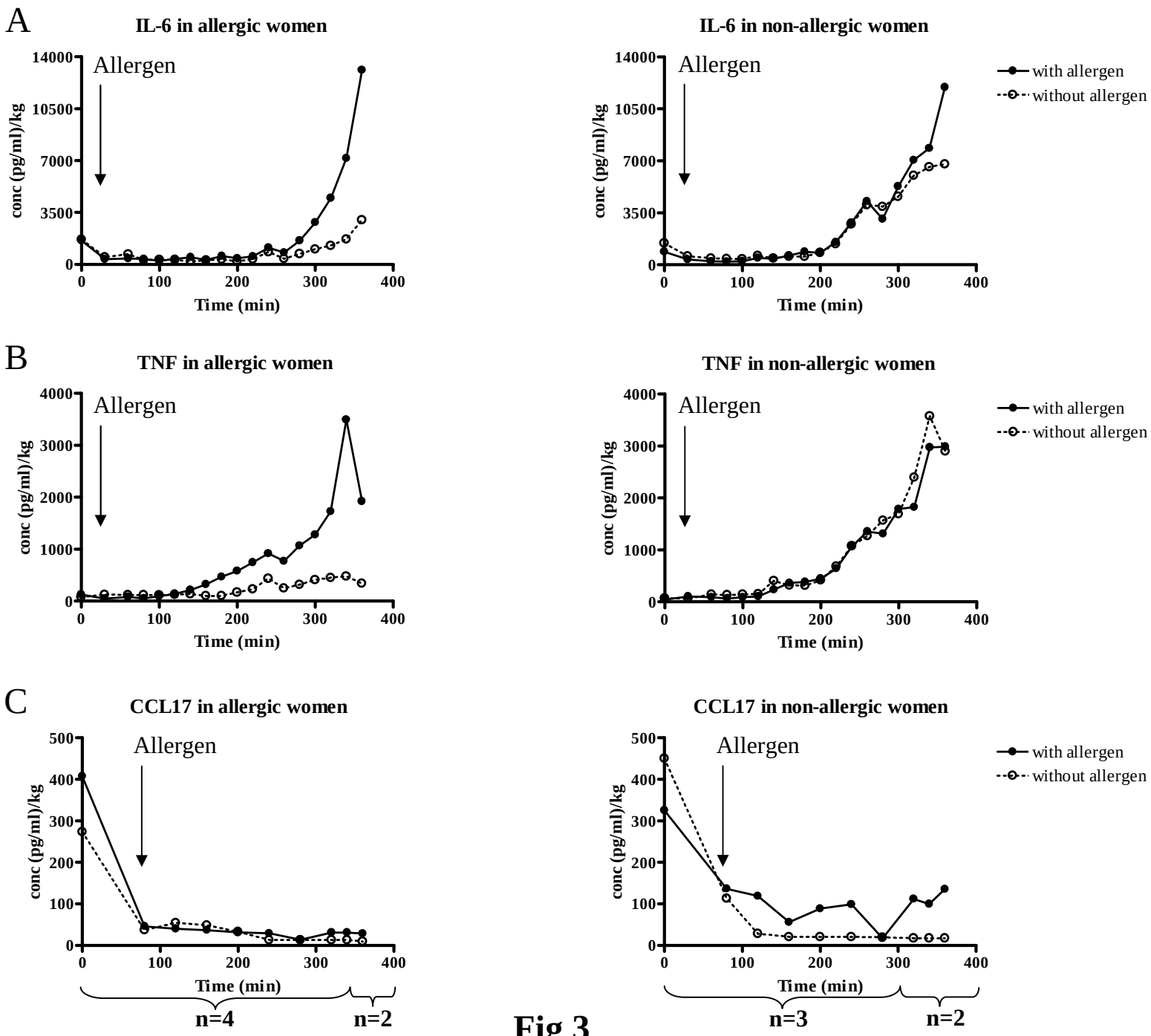
A
IL-6 in allergic women
IL-6 in non-allergic women
Allergen
conc (pg/ml)/kg
Time (min)
with allergen
without allergen
B
TNF in allergic women
TNF in non-allergic women
C
CCL17 in allergic women
CCL17 in non-allergic women
n=4
n=2
n=3
n=2

Fig 3